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T E C H N I C A L R E P O R T

TO

OFFICE OF NAVAL RESEARCH
UNITED STATES DEPARTMENT OF THE NAVY

BIOLOGICAL SCIENCES DIVISION
CLINICAL BRANCH
LT. COMMANDER PAUL LINDSAY, HEAD

FROM

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"PLANT COLLOIDS FOR EVALUATION AS PLASMA EXTENDERS AND FOR COMPLEXING WITH PROTEIN SPARING SUBSTANCES"

REPORT NO. ELEVEN

URS F. NAGER

APRIL 1, 1953

Synthetic Blood Plasma Extenders

Introduction

Work was continued during March on the preparation of propylene glycol and glycerol pectates from pectic acid and the appropriate oxides. Emphasis was placed to avoid excessive amounts of the oxide reagent by esterification in inert, organic media.

As an alternative, Pectic N.F., the purest commercial pectin available, was investigated as starting material for a plasma extender. Esterification of the free carboxyl groups of Pectin N.F. followed by depolymerization of the neutral pectate ester was expected to furnish a new pectin derivative with satisfactory oncotic properties.

Summary

Studies were continued on the heterogenous esterification of pectic acid. Organic solvents were investigated as substitutes for excessive amounts of esterifying agents. Esterification of pectic acid with propylene oxide was shown to occur in the presence of pentane, ether and methanol, but no reaction was obtained in acetone, dioxane, and isopropanol.

By using ether or pentane as reaction media the amount of propylene oxide could be reduced from 9 to 2 times the stoichiometric requirement. This modification not only meant a substantial saving of propylene oxide but also reduced the chances of forming polyglycol pectates. Esterification proceeded considerably more slowly in methanol as demonstrated on a larger scale run. There seemed to be no apparent difference between propylene glycol pectates prepared in pentane, ether, methanol or in propylene oxide alone.

Applying the foregoing observations to the synthesis of glycerol pectate, successful esterification was achieved in methanolic suspension. Again, less glycidol was required compared to previous esterifications in aqueous phase. Esterification attempts in ether yielded resinous reaction products which discouraged further investigation.

Pectin N.F., the purest commercially available pectin, was investigated as a potential source for a plasma extender. A three-step process was considered to convert Pectin N.F. into a suitable oncotic agent: (a) liberation of the unesterified carboxyl groups from the sodium salt, (b) their esterification with either propylene oxide or glycidol and (c) depolymerization.

Removal of inorganic constituents and liberation of unesterified carboxyl groups of Pectic N.F. was accomplished by an acid-alcohol

rinse as shown in two examples. Esterification of the free COOH groups with propylene oxide occurred very readily in suspensions of ether, pentane or excess of reagent. An esterification attempt made in aqueous phase failed because the reaction mixture set up to a firm gel.

In similar manner, glycerol pectate N.F. was prepared by reacting pretreated Pectin N.F. with glycidol in suspensions of ether or methanol.

Depolymerization of esterified Pectin N.F. to material of suitable viscosity and oncotic pressure was accomplished by thermal degradation in aqueous solution. Titrations before and after degradation revealed no saponification of ester linkages.

Additional amounts of glycerol pectate were prepared and submitted to Dr. F. W. Hartman.

The author has consulted and collaborated with Dr. Rene Jennen on various problems relative to this project.

Experimental

I. Heterogenous Esterification of Pectic Acid in Organic Solvents

It has been demonstrated previously that esterification of pectic acid with propylene oxide proceeded equally well in aqueous and non-aqueous media. For complete esterification in an aqueous medium, about 9 times the theoretical amount of propylene oxide was required since substantial amounts of the reagent were lost by a concurrent reaction, probably hydrolysis to propylene glycol. In the heterogenous esterification of pectic acid also a minimum of about 9 times the theory of propylene oxide was employed, most of which was required for adequate suspension of the pectic acid. Although it was not actually determined, a substantial amount of the oxide is believed to be recoverable from this reaction mixture.

Also, in the case of glycerol pectate an excess of glycidol (about 6 moles per equivalent of pectic acid) was required for complete esterification in aqueous solution. As glycidol is not as readily available as propylene oxide, it appeared essential to investigate esterification methods more economical with respect to esterifying agent. Improvements along this line were expected, (a) by using the least amount of water in the reaction mixture and (b) by employing inert solvents as suspending agents for pectic acid.

Organic solvents as substitutes for excessive amounts of reagent were first studied in the preparation of propylene glycol pectate. When found satisfactory, the results were then applied to improving the

synthesis of glycerol pectate.

A. Reacting Pectic Acid with Propylene Oxide in Presence of Organic Solvents

The following exploratory runs were made in 8-ounce beverage bottles charged as shown in the table below. The reaction temperature was 35°C. and the reaction period 16-20 hours.

TABLE I

Run #	Pectic Acid g	Propylene Oxide ml	$R(\frac{PO}{PA})$*	Ether** ml	Pentane ml	H_2O ml
166-1	10	30	9	--	--	2
166-3	10	10	3	20	--	2
166-5	10	10	3	--	20	2
168-8	20	15	2	80	--	2
168-9	20	15	2	--	80	2
166-4	10	5	1.5	25	--	2

* Stoichiometric ratio of propylene oxide (PO) to pectic acid (PA).
** Treated with $FeSO_4$ to remove peroxides.

All but the last bottle (run No. 166-4) showed complete or nearly complete esterification after 16 to 20 hours. Consequently, it followed that the ratio of propylene oxide could be reduced from 9 to 2 times the theory using ether or pentane as suspending agent. There seemed to be no obvious difference in the pectate esters isolated from these solvents.

Additional solvents were tested in the next series of runs made with a basal charge consisting of 20g Pectic Acid #75, 20 ml propylene oxide and 2 ml H_2O. Propylene oxide was thus present in three times the theoretical amount. To this basal charge was added 80 ml solvent such as dioxane, acetone, methanol and isopropanol. The bottles were incubated as above at 35°C and sampled at intervals of 24 hours.

At the end of 72 hours, the runs made with dioxane, acetone and isopropanol showed practically no ester formation. The reaction products were incompletely water soluble, exerted strongly acid reaction and formed insoluble precipitates with calcium ions.

Contrary to this, gradual esterification did occur in the presence of methanol. After incubation at 35°C for 72 hours, the solids had about tripled in volume, were completely water soluble and were no more precipitable with calcium ions.

In the following experiment heterogeneous esterification of pectic acid with propylene oxide in methanol was repeated on a larger scale. Two 32-ounce bottles were charged, each with 100g Pectic Acid #75, 400 ml methanol, 100 ml propylene oxide and 10 ml H_2O. The temperature of the bath in which the bottles were tumbled was 40°C. A sample taken after 16 hours was partially water soluble, reacted strongly acid and yielded a precipitate with calcium ions. At 43 hours, the solids were water soluble, showed still slight acid reaction but were no longer affected by calcium ions. After 64 hours, the reaction mixtures were about neutral. The fact that there was still a substantial amount of propylene oxide left over was rather unexpected. It indicated that the secondary reaction of propylene oxide with methanol to form an ether did not occur to the extent anticipated.

From the combined reaction mixtures the pectate ester was isolated by filtration, rinsing with methanol and acetone, and drying at 70°C for 5 hours. Yield: 193.5 g 2-hydroxypropyl pectate, Lot #P-177.

B. Esterifying Pectic Acid with Glycidol in Presence of Organic Solvents

All the glycerol pectate prepared to date was obtained by reacting pectic acid with glycidol in aqueous solution. Attempts to prepare this ester in a heterogeneous system were postponed pending to the availability of an inert, organic solvent as reaction medium. The results of the esterification experiments with propylene oxide reported in the preceding section suggested the study of the use of solvents, particularly ether and methanol, for the preparation of glycerol pectate. Pentane was not tested because it is immiscible with glycidol.

The following experiments were carried out in 8-ounce beverage bottles with Pectic Acid #75 and redistilled glycidol. The respective charges together with the reaction conditions are shown in Table II.

TABLE II

Run #	Pectic Acid g	Glycidol g	H_2O ml	Solvent	ml	Reaction Time hrs.	T °C
167-2	10	10	2	Ether	20	16	35
168-7	10	10	--	Ether	40	90	40
173-5	10	10	--	Acetone	40	72	35
173-6	10	10	--	Dioxane	40	72	35
177-3	10	10	--	MeOH	40	64	40

The analysis of the reaction products isolated from this series showed the accomplishment of partial esterification in presence of ether and methanol. The reaction products formed in ether consisted of dark amber resins. They were incompletely water soluble and precipitable with calcium ions. Reducing the amount of water in the initial charge (Run #168-7) did not prevent the formation of a sticky, hygroscopic material. The nature of this material discouraged further investigation at this point.

Run #177-3, using methanol as reaction medium, yielded non-hygroscopic material which was easily recoverable. It was readily water soluble, reacted neutral and was not precipitated with calcium ions. No evidence of esterification was found in the presence of acetone or dioxane.

The results of the foregoing, exploratory studies suggest further work on the heterogeneous esterification of pectic acid with glycidol in methanol.

II. Studies with Pectin, N.F.

A different approach leading to a suitable plasma extender based on pectin was the object of the following studies with Pectin N.F., the purest commercially available pectin. It is a partially saponified methyl polygalacturonate with an average molecular weight of about 200,000 to 300,000. Approximately 30% of the carboxyl groups are free or in the form of a salt. If all the COOH groups were esterified with methanol it would no longer be water soluble.

Pectin, N.F. has been used by Dr. F. W. Hartman and others as a starting material for pectin sols as plasma extenders. The treatment consisted in thermal degradation to an approximate average molecular weight of 40,000 effected by autoclaving at 250°F for about 45 minutes. Although successful when used in reasonable quantities, these parenteral sols failed under the severe bleeding tests calling for up to 85%

replacement of blood. It should be emphasized that these sols required neutralization of the free carboxyl groups as well as considerable buffering prior to injection. The high electrolyte content or the ion exchange properties of the unesterified COOH groups may have unbalanced the electrolyte equilibrium of the blood stream. The calcium level was undoubtedly affected because of the known affinity of polygalacturonic acid for calcium, iron, etc.

It was therefore assumed that by esterification of the free carboxyl groups of Pectin N.F. with propylene glycol or glycerol followed by thermal degradation a more suitable material should be readily attainable. If sufficiently esterified it would be neutral and be free of electrolytes and ion exchange properties.

The preparation of this modified pectin involves three steps: (a) removal of inorganic salts from Pectin N.F. by an acid-alcohol rinse, (b) esterification of the free carboxyl groups and (c) thermal degradation of the ester to a suitable molecular size.

A. Pretreatment of Pectin N.F.

The removal of inorganic constituents from Pectin N.F. was accomplished by an acid-alcohol rinse similar to the batchwise regeneration of a cation exchange resin. The alcohol concentration was chosen such as to prevent dissolution of pectin.

In the first run, 200 grams Pectin N.F. was contacted in two successive treatments, each with 1000 ml 75% aqueous isopropanol containing 20 ml conc. HCl, by agitating for 15 minutes and separating by filtration. Excess HCl was then removed by reslurrying twice in 1000 ml 75% isopropanol for 15 minutes and filtering in between treatments. The solids were then dehydrated with 600 ml acetone and finally dried in vacuo over KOH. Yield: 175 grams acid washed Pectin N.F., Lot #P-158.

A second run made with 1000 grams Pectin N.F. in analogous manner yielded 974 grams purified Pectin N.F., Lot #P-174. This preparation showed a neutralization equivalent of 1.47 milliequivalent per gram corresponding to about 28% free COOH groups. A 1% solution showed a pH of about 3.

B. Esterification Attempts with Propylene Oxide

The following experiments were conducted with a view toward developing methods of esterifying the free carboxyl groups of Pectin N.F. Information gained from exploratory studies with propylene oxide was to be applied to subsequent investigations with glycidol.

Esterification was first attempted in aqueous solution. An 8-ounce pressure bottle was charged with 20 grams pretreated Pectin N.F., Lot #P-158, 60 ml propylene oxide, 100 ml H_2O and tumbled at 35°C for 25 hours. A firm gel resulted dissolving to a viscous, neutral solution on addition of water. There was no precipitate formed in presence of calcium ions. Thus, esterification had obviously taken place. The fact that the reaction mixture set to a gel discouraged further studies on homogeneous esterification.

Experiments concerning heterogenous esterification are listed according to the solvents employed.

Propylene oxide. An excess of propylene oxide served here as solvent. A 32 ounce beverage bottle was charged with 100 grams pretreated Pectin N.F., Lot #P-158, 300 ml propylene oxide and 30 ml water. Reaction started quite rapidly requiring cooling under running water for one-half hour. The bottle was then tumbled in a bath at 35°C for 25 hours. Esterification was complete as indicated by the usual tests. The solids were isolated by filtration, rinsing with acetone and drying in vacuo over KOH. Yield: 115.5 grams esterified Pectin N.F., Lot #P-159.

Pentane. The charge of this run consisted of 100 grams pretreated Pectin N.F., 100 ml propylene oxide, 200 ml pentane, and 20 ml H_2O. After reaction at 40°C for 19 hours esterification was complete. The solids were recovered by filtration, rinsing with acetone and drying in the usual manner. Yield: 109 grams esterified Pectin N.F., Lot #P-175-2.

Ether. Except for substitution of pentane by ether (peroxide free) the charging, reaction conditions and isolation were the same as in the preceding run. Yield: 109 grams esterified Pectin N.F., Lot #P-175-1.

The usual tests performed on the solids showed that a high degree of esterification was achieved in the three runs. Unlike the reaction products from esterification in solution, esters of Pectin N.F. obtained by the heterogenous method were very easy to handle and to isolate. Furthermore, a substantial saving of esterifying agent was achieved using pentane or ether without unfavorable effect on the reaction rate.

In summary, the preferred method of preparing propylene glycol esterified Pectin N.F. consists in reacting a mixture of 5 parts pretreated Pectin N.F., 5 parts propylene oxide, 10 parts pentane, and 1 part H_2O at 40°C for 20 hours.

C. Esterification Attempts with Glycidol

Esterification was observed to occur in aqueous solution, i.e. by reacting a charge consisting of 20 grams pretreated Pectin N.F., 50 grams glycidol, and 90 ml H_2O at 35°C for 25 hours. Early during the reaction however, the mixture set to a semi solid mass and could no longer be agitated. Again, the superior route appeared to be heterogenous esterification in presence of methanol or ether as reaction media.

Methanol. An 8-ounce beverage bottle was charged with 20 grams pretreated Pectin N.F., Lot #P-174, 10 grams glycidol, 40 ml methanol, and 2 ml H_2O. The sample was reacted in the tumbler at 40°C for 62 hours. The solids were isolated by filtration, rinsing successively with methanol and acetone, and drying. Yield: 22 grams glycerol esterified Pectin N.F., Lot #P-178. This material required less than 0.1 meq per gram alkali for neutralization which corresponds to an ester content in excess of 98%. It showed a relative viscosity of 18.0 in water at 1% concentration.

Ether. A charge consisting of 20 grams pretreated Pectin N.F., Lot #P-174, 20 grams glycidol, 40 ml ether (peroxide free), and 4 ml H_2O was reacted at 40°C for 19 hours. The solids isolated in the usual manner weighed 26 grams. This preparation, designated Lot #P-176, was only partially water soluble. It proved to be neutral and unaffected by calcium ions. It was quite thermostable in the pH range of 1 to 7 but dissolved by adding a small amount of caustic.

The conclusion of the above mentioned experiments was that heterogenous esterification of Pectin N.F. could be achieved in methanol or ether with a lower ratio of glycidol.

D. Thermal Degradation of Esterified Pectin N.F.

According to viscosity data, the average molecular weights of Pectin N.F. and ester derivatives prepared thereof were in the range of about 200,000 to 300,000. It was therefore necessary to depolymerize these materials in order to obtain preparations of desired viscosity and oncotic pressure. Thermal degradation as a means of depolymerization was the subject of the following studies.

Pectin N.F. Commercial Pectin N.F. (40 grams) and NaCl (10 grams) were dissolved in 2000 ml water. Celite, analytical grade filter aid (60 grams), was added and the mixture autoclaved 45 minutes at 15 pound steam pressure. The reaction mixture was filtered, while still hot, through a clarifying pad yielding a sparkling clear filtrate designated #157. Relative viscosity and oncotic pressure are given in Table IV.

No satisfactory method was found to date to isolate the degraded Pectin N.F. On treating solution #157 with solvents such as acetone, methanol or isopropanol fine, gelatinous precipitates resulted which showed no tendency to settle out. Recovery by filtration was unsuccessful. Addition of NaCl greatly reduced the volume of the precipitate and effected transformation of the gels into solids. Isolation by filtration, rinsing and drying was now possible but the final materials were heavily contaminated with NaCl.

Propylene glycol pectate. Propylene glycol pectate N.F., Lot #P-159 (40 grams) and NaCl (10grams) dissolved in 2000 ml water were autoclaved 45 minutes at 15 pounds steam pressure. Filtration was accomplished with the aid of 60 grams Celite and a clarifying pad. A sparkling clear filtrate was obtained designated as #165. Relative viscosity and oncotic pressure are given in Table IV.. Potentiometric titration of a 100 ml aliquot:

TABLE III

1-N NaOH ml	pH
0	3.9
0.1	4.4
0.15	5.2
0.18	7.0

Degraded propylene glycol pectate N.F., Lot #165 differed from the degraded native Pectin N.F., Lot #157 by having a higher relative viscosity and lower oncotic pressure. The esterification of carboxyl groups was already shown to be responsible for this. Most important however was the observation that the ester linkages remained practically unaffected as evidenced by the titration data.

The effect of longer degradation was studied next. Propylene glycol pectate N.F. P-175-1 (15 grams) was dissolved in 750 ml H_2O and autoclaved with 30 grams Celite for 1¼ hours at 15 pounds steam pressure. The filtrate #179-1 showed a lower viscosity but very little gain in oncotic pressure (see Table IV).

The combination of higher initial concentration and prolonged degradation time finally yielded material, meeting Dr. Hartman's specifications for a plasma extender. Propylene glycol pectate N.F. P-175-1 (60 grams) dissolved in 2000 ml H_2O was autoclaved with 90 grams Celite for 1½ hours at 16 pounds steam pressure. The filtrate of this reaction mixture was a clear, light amber solution #180. It

had the best oncotic pressure and relative viscosity of the series (see Table IV).

TABLE IV

Run #	Material	Conc. %	Degradation hrs.	η rel.*	Oncotic Pressure mm H_2O** 24	48	72	96 hrs.
157	Pectin N.F.	2	3/4	6.3	275	530	670	780
165	Propylene Glycol Pectate N.F.	2	3/4	9.5	160	210	255	295
179-1		2	1-1/4	3.8	131	200	265	320
180		3	1-1/2	5.2	165	280	380	470
179-2	Glycerol Pectate N.F.	2	1-1/4	3.8	100	160	220	260

* Relative viscosity at 25°C.
** Determined in Saline against H_2O.

Glycerol pectate. Glycerol pectate N.F. P-176 (22 grams) (prepared in ether suspension) and Celite (44 grams) were autoclaved with 1100 ml H_2O at 16 pounds for 1¼ hours. A clear, light amber filtrate #179-2 was obtained, having a pH of 5. As mentioned earlier in this report, preparation P-176 was not completely water soluble. Therefore, it was questionable wether the insoluble fraction underwent dissolution and thermal degradation. Subsequent degradations of soluble glycerol pectate (prepared in methanol suspension) have yielded higher viscosities and oncotic pressures than obtained with the above ester P-176. The results are to be reported next month.

III. Preparing Glycerol Pectate Lots No. 4 and 5

The following batches of glycerol pectate were prepared upon request of Dr. Hartman for additional material of a low and a high molecular weight fraction for comparative evaluation in vivo. It was essentially a duplication of the previous batches Nos. 2 and 3 (Report No. 10).

Three 32-ounce beverage bottles were charged, each with 75 grams Pectic Acid #75, 150 grams glycidol, and 340 ml H_2O and reacted 24 hours at 35°C. The combined reaction mixtures were bleached with ClO_2, filtered and clarified. Crude glycerol pectate was precipitated by

adding an equal volume of acetone. The precipitate was rinsed by slurrying and decanting with 75% acetone.

The washed glycerol pectate was redissolved in water and reprecipitated with acetone. As before, a mixture of precipitated and emulsified material was obtained. The solids were filtered off, rinsed and dried in the usual manner. Yield: 81 grams glycerol pectate, Lot #4.

The ester present in the filtrate was coagulated with saturated NaCl solution and gave, after rinsing and drying as usual, 120 grams glycerol pectate, Lot #5. Following are the viscosities and oncotic pressures of glycerol pectate, Lots No. 4 and 5.

TABLE V

Lot No.	η rel. C=4%	η rel. C=2%*	Pressure mm H_2O** 24	48	72	144 hrs.
4	10.0	3.38	120	175	230	320
5	5.82	2.48	140	205	275	365

* In Saline.
** C=2% in Saline against H_2O.

The preparations, Lots No. 4 and 5 of glycerol pectate, are currently being tested by Dr. F. W. Hartman at Henry Ford Hospital Laboratories.